AD-A216 667

SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

DTIC ELECTE JAN 09 1990 S D

1a. REPORT SECURITY CLASSIFICATION: (U)	1b. RESTRICTIVE MARKINGS: NA
2a. SECURITY CLASSIFICATION AUTHORITY: NA	3. DISTRIBUTION/AVAILABILITY OF REPORT: Distribution Unlimited
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE: NA	
4. PERFORMING ORGANIZATION REPORT NUMBER(S): NA	5. MONITORING ORGANIZATION REPORT NUMBER(S): NA
6a. NAME OF PERFORMING ORGANIZATION: Univ. of Illinois; 6b. OFFICE SYMBOL (If applicable): NA	7a. NAME OF MONITORING ORGANIZATION: Office of Naval Research
6c. ADDRESS (City, State, and ZIP Code): 506 South Wright Street, Urbana, IL 61801	7b. ADDRESS (City, State, and ZIP Code): 800 N. Quincy Street, Arlington, VA 22217-5000
8a. NAME OF FUNDING/SPONSORING ORGANIZATION: Office of Naval Res.; 8b. OFFICE SYMBOL (If applicable): ONR	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER: N00014-88-K-0093
8c. ADDRESS (City, State, and ZIP Code): 800 N. Quincy Street, Arlington, VA 22217-5000	10. SOURCE OF FUNDING NUMBERS: PROGRAM ELEMENT NO. 61153N; PROJECT NO. RR04106; TASK NO. 442S005; WORK UNIT ACCESSION NO.

11. TITLE (Include Security Classification): (U) Characterization of Biofilm Community Structure by Ribosomal RNA Sequences

12. PERSONAL AUTHOR(S): Stahl, David A.

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Annual	FROM 12/1/88 TO 11/30/89	Dec. 1, 1989	6

16. SUPPLEMENTARY NOTATION: NA

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	
06	03		Biofilm, ribosomal RNA, oligonucleotide hybridization probes, spatial distribution, fluorescent DNA probes, in situ hybridization, (continued)

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

The general objectives of this project are to develop molecular techniques, based upon comparative ribosomal RNA sequencing, for the explicit identification and localization of individual microorganisms in natural biofilms. As model systems sulfidogenic and methanogenic fixed-bed bioreactors have been established. Sampling protocols for microscopic and molecular analyses of, and methods for measuring methane production and sulfate reduction within, these bioreactor systems were developed. The use of fluorescent-oligonucleotide probes for visualizing individual sulfate-reducing bacteria within complex biofilms was demonstrated. The polymerase chain reaction was used for the selective amplification of 16S rRNAs from defined physiological assemblages of organisms (methanogens and sulfate-reducing bacteria). The cloning and sequencing of these amplified products has provided an outline of the microbial (continued)

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION	
☒ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT. ☐ DTIC USERS	(U)	
22a. NAME OF RESPONSIBLE INDIVIDUAL: M. Marron	22b. TELEPHONE (Include Area Code): 202-696-4760	22c. OFFICE SYMBOL: ONR

DD Form 1473, JUN 86 — Previous editions are obsolete. — SECURITY CLASSIFICATION OF THIS PAGE

S/N 0102-LF-014-6603

18. comparative sequencing, phylogeny, sulfate-reducing bacteria.

19. diversity within the sulfidogenic and methanogenic bioreactors. Comparative sequencing of additional strains of marine and freshwater caulobacters has been completed in association with ONR investigator John Smit. The use of flow cytometry to enumerate a target organism, in a defined mixture of cells, using a genus-specific fluorescent probe was demonstrated.

ANNUAL REPORT ON CONTRACT N0014-88-K-0093 R & T CODE 442s005---01

PRINCIPAL INVESTIGATOR: David A. Stahl

CONTRACTOR: University of Illinois

CONTRACT TITLE: Characterization of Biofilm Microbial Community Structure by Ribosomal RNA Sequences

RESEARCH OBJECTIVE: Develop molecular techniques for the explicit identification and localization of microorganisms comprising natural biofilms developed in fixed-bed bioreactors. To use natural consortia containing sulfate-reducing and methanogenic bacteria to evaluate the techniques and to study the effects of changing reactor conditions (e.g. sulfate concentration) on community composition and the spatial relationships among the resident microorganisms. Long term objectives include the identification and characterization of obligate symbioses within fixed consortia and the autecological basis for community function.

PROGRESS (Year 2): During second year of the project, replicate fixed-bed bioreactors (favoring either methanogenesis or sulfate reduction) were established. Protocols for measuring methane production and sulfate reduction have been developed and methane and sulfate concentrations in the four bioreactor systems constantly monitored over the past three month period. Based on these results the bioreactor feed composition has been adjusted to balance oligotrophic conditions (30 mg Carbon/L) with reproducible measurements of methane and sulfate concentrations.

Fixation and hybridization conditions for in situ hybridization of intact single cells were established. Enzyme-conjugated (horseradish peroxidase) and fluorescent-dye-conjugated oligonucleotide hybridization probes were synthesized for initial comparative studies during the first project year.
Most effort during the first and second years was directed to the characterization of fluorescent dye-conjugated probes for distinguishing various groups, genera, species and subspecies of bacteria. Probes synthesized and evaluated for specificity included 1) genus, species- and subspecies-specific probes for strains of Fibrobacter, 2) Desulfobacter genus-specific probe, 3) Desulfosarcina genus-specific probe, 4) archaebacterial kingdom-specific probes and 5) eubacterial kingdom-specific probes 5) eukaryote kingdom-specific probe and 6) a general probe encompassing all characterized sulfate-reducing bacteria. The Fibrobacter spp. probes were fabricated for establishing the resolving power (e.g. species vs. subspecies discrimination) of whole-cell hybridization. Single mismatch discrimination between probe and target sequence for determinative fluorescence microscopy was demonstrated for certain of the probes evaluated.

Earlier apparent difficulties with fluorescent probe penetration into established biofilm communities have been resolved. Individual sulfate-reducing bacteria have now been visualized within intact complex biofilms by the combined use of fluorescent-oligonucleotide probing and fluorescence microscopy (Figure

1). Preliminary studies with the confocal laser scan microscope suggest that this system could offer the basis for reconstructing the three dimensional structure (organism by organism) of biofilm communities.

Group-specific Amplification and Cloning. The polymerase chain reaction has been used to selectively amplify common regions of the 16S rRNA from phylogenetically-defined assemblages of organisms for cloning and sequencing. One universal PCR primer set (for amplifying a common region of the 16S rRNA from any organism) and two group-specific primer sets have been fabricated. The group-specific primer sets selectively amplify either sulfate-reducing bacteria or archaebacteria.

The SRB-specific primers have so far identified two species of sulfate-reducing bacteria in our sulfidogenic bioreactors. Comparision of the cloned sequences to our reference collection has shown one clone to be closely related to Desulfovibrio vulgaris (ca 95% similarity) and the other to Desulfuromonas acetoxidans (ca 95% similarity). These relationships are close enough to infer the basic physiology of these organisms, although neither have been previously described in pure culture. Analysis of the universal primer set derived clones is in progress (ten unique clones have so far been sequenced).

Nucleotide sequence determinations of five additional strains of caulobacter are in progress (ten total). The caulobacter characterizations are part of a collaborative research project with ONR investigator John Smit assessing the natural diversity among freshwater and marine varieties of these sessile bacteria and has shown them to be a genetically diverse, yet phylogenetically coherent, assemblage. The sequence information will be used as the basis for developing group- and species-specific oligonucleotide probes for hybridization studies of natural biofilms.

Flow cytometry. Work recently completed in association with investigators at The Massachusetts Institute of Technology (B. Binder and S.W. Chisholm) and Woods Hole Oceanographic Institution (R. Olson) has demonstrated the feasibility of using fluorescent oligonucleotide probing to specifically tag cells for enumeration by flow cytometry. Further development of the technique should permit the rapid assessment of group or species composition of planktonic communities.

ACCOMPLISHMENTS: Demonstration of specific labeling and microscopic identification of individual sulfate-reducing bacteria within intact complex biofilms. Development of group-specific amplification, cloning and sequencing protocols for selective recovery of sulfate-reducing bacteria rRNA sequences and other defined phylogenetic assemblages from biofilm microorganisms. Demonstration of the use of the probing methodology for the identification and enumeration of selected bacteria by flow cytometry.

REFERENCES: none

WORKPLAN (Year 3): The third year will continue essentially as outlined in the original proposal. Community structure of the methanogenic and sulfidogenic

bioreactor systems will continue to be evaluated by comparative sequence analysis and DNA probing. The reactors will then be perturbed by altering the availability of sulfate (by addition of sulfate to the methanogenic reactor and removal of sulfate from the sulfidogenic reactor). Community response to changing sulfate concentration will be measured by DNA probing. Change in the spatial distribution of specific biofilm organisms will be evaluated by fluorescent-oligonucleotide probing in combination with fluorescence microscopy. Specific associations (symbioses) suggested by concerted population shifts (as determined by DNA probing) will be microscopically characterized by the use of species-specific fluorescent probes. A graduate student from environmental engineering will evaluate the feasibility of developing mathematical models for predicting the function and community composition of these bioreactor systems.

ONR SPONSORED PUBLICATIONS

In Press Refereed Journals

Amann, R., L, Krumholz, and D.A. Stahl. Fluorescent Oligonucleotide Probing of Whole Cells for Determinative, Phylogenetic and Environmental Studies in Microbiology. J. Bacteriol. (in press).

Book Chapters

Walch, M., W.A. Hamilton, P.S. Handley, N.C. Holm, J.G. Kuenen, N.P. Revsbech, M.A. Rubio, D.A. Stahl, O. Wanner, D.M. Ward, P.A. Wilderer, J.W.T. Wimpenny. Spatial Distribution of Biotic and Abiotic Components in the Biofilm. In: Dahlem Workshop Report on Structure and Function of Biofilms. W.G. Characklis and P.A. Wilderer (eds.) John Wiley & Sons (1989).

Submitted or In Press Chapters

Stahl, D.A. and R. Amann. Development and application of nucleic acid probes in bacterial systematics. In: Sequencing and Hybridization Techniques in Bacterial Systematics. E. Stackebrandt and M. Goodfellow (eds.), John Wiley and Sons, Chichester, England.

Invited Presentations at Scientific Conferences

Dahlem Workshop on Structure and Function of Biofilms. Berlin, November 27 - December 2, 1988.

Molecular Approaches to the Study of Environmental Microbiology. Annual Meeting of the American Society for Microbiology. New Orleans, La. May 14 - May 18, 1989.

Chemotaxonomic Approaches for Microbial Ecology. Fifth International Symposium on Microbial Ecology. Kyoto, Japan. Aug. 27 - Sept. 1, 1989.

Papers in Preparation

Amann, R.I., B. Binder, S.W. Chisholm, R. Olson, and D.A. Stahl. Combined use of phylogenetically-based fluorescent hybridization probes and flow cytometry.

Two additional papers are in preparation describing the selective recovery of sulfate-reducing bacterial 16S rRNA from natural biofilms and the microscopic identification of individual species within intact biofilms by fluorescent DNA probing are in preparation.

Graduate Students Supported

Female - 1

Postdoctoral associates Supported

Male - 1

PATENTS/AWARDS/FELLOWSHIPS: none

Figure 1. Phase contrast (A) and fluorescence micrographs (B) of a five day-old biofilm from a sulfidogenic fixed-bed bioreactor. The biofilm was allowed to develop for five days on a glass coverslip placed at the top of the reactor. The coverslip was removed, fixed with formaldehyde and hybridized with the SRB-specific rhodamine-conjugated oligonucleotide. Two distinct morphological types are apparent in the fluorescence micrograph: 1) large vibrios, presumptively identified as Desulfovibrio vulgaris-like organisms and 2) small rods, presumptively identified as Desulfuromonas acetoxidans-like organisms.

Figure 1

A

B